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Characteristics of Muskellunge Spermatozoa I: Ultrastructure of Spermatozoa and Biochemical Composition of Semen

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Abstract. — We investigated the ultrastructure of spermatozoa and some physiological and biochemical parameters of semen in muskellunge *Esox musquinongy.* Ultrastructure of spermatozoa analyzed with scanning and transmission electron microscopes revealed a spherical head, 1.5 µm in diameter. Unlike the spermatozoa of other teleost fishes, an elongated midpiece with abundant mitochondria was found to be characteristic for muskellunge spermatozoa. Three techniques for measuring sperm concentration, counting with a hemacytometer, spermatocrit, and spectropho-tometric methods, provided similar results (19.7 ± 6.1 x 10^9 sperm/mL). The spectrophotometry method is recommended because it is rapid and requires only small amounts of semen. Ionic concentrations and osmolality (289 ± 16.8 milliosmols/kg) of seminal plasma were reported. Sodium (129 ± 6.7 mM) and potassium (27.88 ± 3.27 mM) concentrations were closer to levels found in the seminal plasma of salmonids than cyprinids. Aspartate aminotransferase (AspAT) activity was measured with and without supplement of pyridoxal phosphate (PLP) from sperm extracts. A 96% increase of AspAT was found when PLP was included in the assay. We confirmed the presence of antiprotcinasc activity (against cod trypsin) in seminal plasma of muskellunge. Using the flow cytometry method, we estimated that the DNA stainability of muskellunge spermatozoa was 0.188 ± 0.008 limes that of red blood cells (TRBC) from diploid rainbow trout *Oncorhynchus mykiss.* The DNA stainability of muskellunge spermatozoa was significantly lower than that of muskellunge embryonic cells, which was 0.474 times that of the TRBC (2.21 pg DNA/ cell, based on the internal standard of TRBC of 4.66 pg DNA/cell).

Muskellunge *Esox masquinongy* is one of the most popular sport fishes in the north-central United States. Because of the failure of natural reproduction. annual stocking of this species is frequently required to maintain the population. In recent years, artificial manipulation of muskellunge gametogenesis has received increasing attention. However, only fragmentary information on physiology and biochemistry of spermatozoa (hereinafter, sperm) is available foresocids (Billard 1978; Duplinsky 1982; Koldras and Moczarski 1983). More baseline data are needed to establish sperm quality criteria and for studies of sex manipulation and sperm storage in this species.

Ultrastructure of sperm has been studied in 280 fish species from more than 100 families (Mattei 1991). However, only a brief description of sperm ultrastructure has been made for northern pike *Esoxlucius* (Billard 1970). Gwo and Arnold (1992) described the morphological changes of sperm after cryopreservation in Atlantic croaker *Micropogonias undulatus.* Further information on ultra-structure is needed for understanding sperm biology in esocids, which may be invaluable in developing sperm cryopreservation methods.

A quick and accurate assessment of sperm concentration is required for fertilization experiments and sperm cryopreservation. Sperm concentration assessed with a hemacytometer is widely used to measure sperm quality in fish. Spermatocrit (Bouck and Jacobson 1976) and spectrophotometric methods (Billard et al. 1971; Suquet et al. 1992; Ciereszko and Dabrowski

1993) have been used as alternative methods to estimate sperm density.

Sperm of most teleost fish are quiescent in seminal plasma and are activated after being released into water. Potassium concentration and osmolality have been demonstrated to be the major factors that control the motility of salmonid and cyprinid sperm, respectively (Morisawaet al. 1983a, 1983b). We demonstrated that sperm motility in muskellunge was mainly regulated by osmolality and that calcium had an inhibitory effect (Lin and Dabrowski 1996). Thus, a determination of both the ionic concentration and osmolality of seminal plasma is important in this species. Cruea (1969) found that chloride concentration was much lower (91.3 mM) in seminal plasma of northern pike *Esox hicius* than in salmonids (156-172.6 mM).

Activity of aspartate aminotransferase (AspAT, enzyme number 2.6.1.1) is a reliable biochemical indicator for mammalian and fish sperm quality (Brown et al. 1971; Ciereszko et al. 1990; Ciereszko and Dabrowski 1994). A significant correlation has been demonstrated between AspAT activity and sperm motility and fertilization rate in rainbow trout (Ciereszko and Dabrowski 1994). Changes in AspAT activity were observed in each step of sperm cryopreservation, and the activity has been used as an index of cryogenic damage in sperm and of their fertilizing ability in bovines (Pace and Graham 1970). The extent of disruption of sperm cells or changes in the membrane permeability due to storage or overmaturation could be monitored by the leakage of AspAT into plasma enzymes (Ciereszko and Dabrowski 1994). Proteinase inhibitor(s) found in the seminal plasma of several teleost species raise the question of the regulatory function of this protein in fish taxa that have anacrosomal sperm (Dabrowski and Ciereszko 1994).

In this study, we investigated the ultrastructure of sperm and evaluated three methods of estimating sperm concentration. We analyzed protein and ionic concentrations and osmolality of seminal plasma, AspAT activity of sperm extracts, and antiproteinase activity of seminal plasma. We also estimated the DNA content of muskellunge sperm by the flow cytometry method.

Methods

Collection of samples. — Muskellunge males, 3-5 years of age (703-991 mm total length), were captured by trap nets from the Clear Fork Reservoir, Ohio, during the spawning seasons (April-May) of 1993 and 1994. Semen was stripped from un-anesthetized fish by abdominal pressure and collected into a vial. Care was taken to avoid contamination of the semen sample with blood and urine. Semen was stored on ice for up to 6 h before being analyzed. Semen was also collected from fish maintained in ponds at the Minor E. Clark Fish Hatchery, Kentucky. Four of those semen samples were used for flow cytometry measurement of DNA content.

Ultrastructure of sperm. — Fresh semen was fixed with a paraformaldehyde-glutaraldehydeosmium tetroxide mixture, following the method of Lahnsteiner and Patzner (1991). The specimens were dehydrated in a graded ethanol series and embedded in EPON 812. Thin sections were stained with uranyl acetate and lead citrate for transmission electron microscopy (TEM). For scanning electron microscopy (SEM), specimens were dehydrated, subjected to critical point drying, and then coated with a film of evaporated gold-platinum. Samples were observed and photographed with a Philips CM 12 electron microscope.

Estimation of sperm concentration. — Sperm density in 19 males was evaluated by procedures described by Ciereszko and Dabrowski (1993). Briefly, sperm counting was done with a Neubauer counting chamber after 1,000× dilution with 0.75% NaCl plus 0.6% KCl. Spermatocrit was determined after centrifugation at 12,500 revolutions per minute (rpm) for 10

min in 75-mm capillary tubes in a Beckman Microfuge 12, equipped with a capillary tube rotor. Optical density of diluted semen (l,000×) was estimated with a Beckman DU-70 spectrophotometer with a wavelength of 610 nm. Also, the effect of wavelengths on the optical density was estimated by scanning through the visible light range. The correlation among optical density at wavelengths of 400, 500, 700, and 800 nm was analyzed by using semen from 11 males.

Determination of protein and ionic concentrations and osmolality of seminal plasma. — Seminal plasma from four individual males was used to estimate ionic concentrations (April 1994). Seminal plasma was collected after centrifugation at 12,500 rpm for 10 min at 4°C and stored at -80°C before analysis. Concentrations of potassium, sodium, calcium, magnesium, phosphate, chloride, and other ions were analyzed by an inductively coupled plasma emission spectrometer (ICP, model ARL-3560, Applied Research Laboratory, Valencia, California). Osmolality of seminal plasma from 10 individual males was measured with a freezing point micro osmometer (μ OSMETTE, Precision Systems, Inc., Natick, Massachusetts). The instrument was calibrated with 100 and 500 milliosmols (mosmol)/kg osmometry standards (Precision Systems, Inc.) before each measurement. Protein concentration was measured according to the Bradford (1975) method with the Coomassie protein assay (Pierce, Illinois) with bovine serum albumin as a standard.

Aspartate aminotransferase activity. — Aspartate aminotransferase activity was measured with a reduced nicotinamide adenine dinucleotide (NADH) method (340 nm), according to Bergmayer et al. (1986). Sperm pellets were stored at -80°C before being analyzed. A subsample of sperm pellets was dissolved in 0.1% triton in 0.1 M tris buffer at pH 8.2 and stored in a refrigerator (4°C) for 1 h. Supernatant was separated through centrifugation at 12,000 rpm for 10 min at 4°C. The sperm extract was incubated at 30°C for 10 min with a reaction mixture, with or without pyridoxal phosphate (PLP), that also contained L-aspartate, NADH, malate dehydrogenase, lactate dehydrogenase (Ciereszko and Dabrowski 1995). Aspartate aminotransferase activity was determined with a spectrophotometer (DU 620, Beckman). Protein concentration of sperm extracts was measured as described above.

Antiproteinase activity of seminal plasma. — Antiproteinase activity of seminal plasma was assayed following the method described by Bergmayer (1983) and Dabrowski and Ciereszko (1994). Inhibition of amidase activity of cod trypsin (Sigma, St. Louis, Missouri) was determined spectrophotometrically with *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as a substrate. The trypsin assay mixture contained 0.25 mM BAPNA, 32-units (U) cod trypsin/L, and 20 mM $CaCl_2$ in 0.1 M tris buffer (pH 8.2), and activity was measured at 25°C.

Measurement of DNA content by flow cytometry method. — Semen of 9 males (4 from the Minor E. Clark Fish Hatchery and 5 from Clear Fork Reservoir) was used in this experiment. Sperm was cryopreserved with an extender containing 0.6 M sucrose and 10% dimethyl sulfoxide (Holtz 1993). Sperm pellets were thawed at room temperature. Sperm suspension (10^7 sperm cells) was added to 1 mL staining solution, containing 50 mg propidium iodide, 10 mg ribonuclease A in 1 L Hematall (Fisher Scientific, Pittsburgh, Pennsylvania). Red blood cells of rainbow trout *Oncorhynchus mykiss* were used as both an external and internal standard. Blood samples to serve as standards were collected from the same individual rainbow trout maintained in our laboratory. Samples were stained overnight and filtered through a 60-μm nylon filter, then measured with an Elite flow cytometer (Coulter, Inc., Hialeah, Florida). We measured fluorescence, forward scatter, and 90-degree scatter to determine DNA content and cell size with the standard Elite workstation software. Data were collected from at least 10,000 events (cells)

per male and used for analysis.

Statistical analysis. — All parameters of sperm were expressed as arithmetical means and standard deviations. One-way analysis of variance (ANO-VA) and student *t*-tests were used in comparisons among groups. Regression among three criteria of sperm density was performed with the StatView 512+™, version 1.2 statistical software program (Abacus Concepts, Inc., Berkeley, California).

Results and Discussion

Ultrastructure of Muskellunge Sperm

Muskellunge sperm consisted of three distinct parts: head, midpiece, and tail (Figure 1). The head was round with a diameter of about 1.5 µm. No acrosome structure was present. The chromatin consisted of many clumps of dense granules surrounded by a nuclear envelope bordering the cytoplasmic membrane (Figure 1). The proximal centriole was perpendicular to the distal centriole. Both centrioles were located within the nuclear fossa and had a conventional 9+0 microtubular triplet construction. The midpiece was an elongated sleeve and was completely separated from the flagellum by an invagination of the cell membrane. Relatively large amounts of mitochondria were scattered throughout the midpiece. The cytoplasmic canal was present. The flagellum had the classical 9+2 axoneme structure. A cytoplasmic expansion (fin) was present on only one side of the flagellum. Ultrastructure of muskellunge sperm was comparable to sperm of northern pike described by Billard (1970). The elongated midpiece and abundant mitochondria of muskellunge sperm requires emphasis. Further investigations are necessary to clarify the possible relation between abundant mitochondria and the long duration of sperm movement in muskellunge.

Estimation of Sperm Concentration

Sperm concentration was $19.7 \pm 6.1 \times 10^9$ sperm cells/mL (range, 7.46-30.0). The spermatocrit was 35.7 ± 10.6% (range, 15.9-54.2%). There were linear relationships among the three parameters of sperm density, as shown in Figure 2. Thus, all three techniques provided similar measurements of sperm density. Spermatocrit could be applied to estimate sperm concentration in muskellunge as has been shown in rainbow trout, yellow perch, *Perca flavescens* and lake whitefish *Coregonus clupeaformis* (Ciereszko and Dabrowski, 1993). However, spermatocrit cannot be used to evaluate sperm concentration in salmonids (Bouck and Jacobson 1976), northern pike (De Montalembert et al. 1980), and turbot *Scophthalmus maximus* (Suquet et al., 1992) due to a lack of correlation. The problem associated with using spermatocrit in muskellunge is that testing required at least 0.2 mL semen, which is a relatively high volume for muskellunge. The normal volume of semen collected from the Clear Fork Reservoir population is about 0.5 mL from a 3-6-kg fish. Based on our results, the spectrophotometric method is recommended for measurement of sperm density in muskellunge. This method is rapid and requires only 10 µL semen. Optical density of sperm suspension declined with increasing wavelength; absorbance was from 0.48 at 320 nm to 0.22 at 900 nm for representative samples. The optical density of seminal plasma was not detectable with the same dilution in muskellunge. The reason optical density declined in sperm suspension is still unknown. Our further examination of optical density at 400, 500, 610, 700, and 800 nm from 11 males showed strong correlations ($r = 1$) among these wavelengths. This result indicated that measurements can be done with any of these wavelengths. However. optical density of sperm suspension only slightly declined in rainbow trout (Ciereszko and Dabrowski 1993). Suquet et al. (1992) reported

changes of optical density of sperm suspension over 320-700 nm and minimal interference of seminal fluid at 420 nm in turbot.

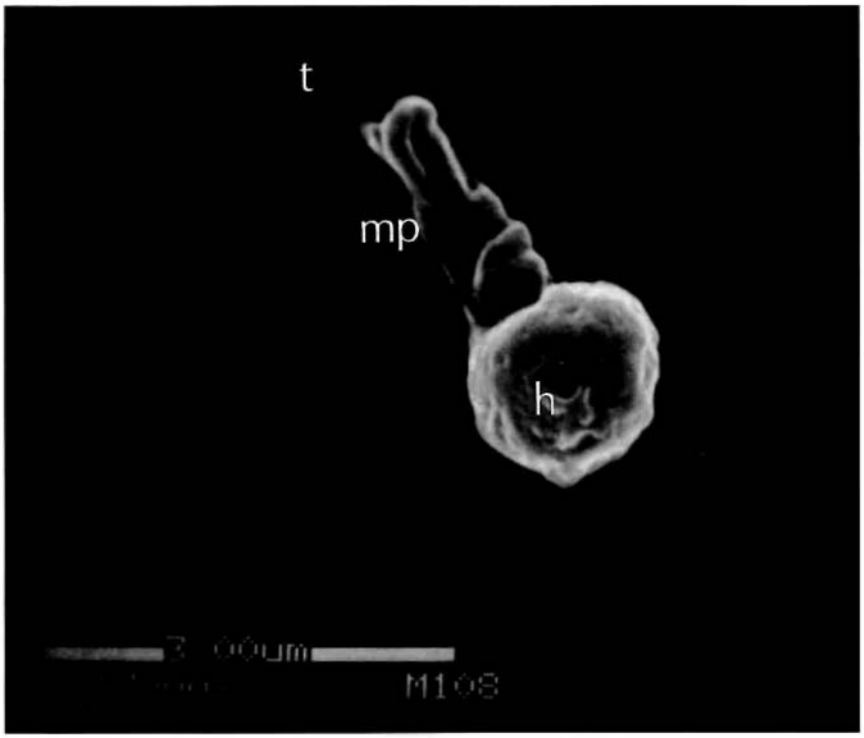

FIGURE 1. — Ultrastructure of muskellunge spermatozoa. The image at the top, generated by a scanning electron microscope shows the head (h). midpiece (mp), and tail (t). Bar = 3.0 µm. The image on the bottom, made by a transmission electron microscope, is of a longitudinal section of the spermatozoa and shows the compact nucleus (n); proximal centriole (pc); mitochondria (ml); theanoxeme (a), showing the usual 9 + 2 pattern: and the cytoplasmic expansion (ce; fin). Bar = 1 µm.

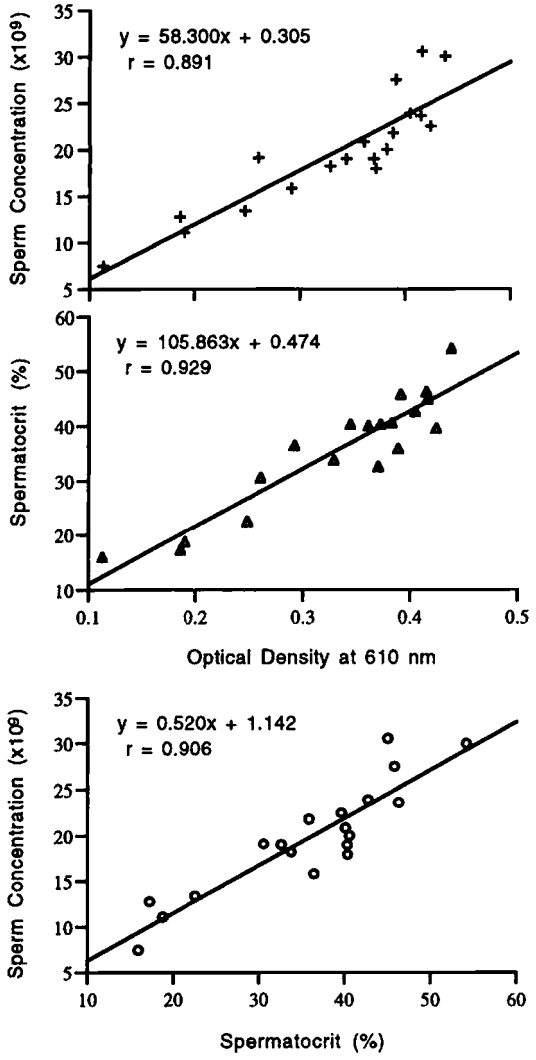

FIGURE 2. — Relationship of indirect measures (optical density and spermatocrit) to sperm density (N = 19). There were significant correlations among all three criteria of sperm density (P = 0.0001).

TABLE 1 — Mean protein and ion concentrations and osmolality of muskellunge seminal plasma.

Constituent or variable	Mean	SD	*N*
Potassium (mM)	27.88	3.27	4
Sodium (mM)	129.17	6.69	4
Calcium (mM)	1.72	0.26	4
Magnesium (mM)	1.05	0.11	4
Phosphate (mM)	7.81	2.17	4
Chloride (mM)	120.32	7.86	4
Protein (mg/ml)	0.538	0.095	10
Osmolality (mosmol/kg)	289.5	16.8	10

Ionic Concentration and Osmolality of Seminal Plasma

Muskellunge seminal plasma showed a sodium concentration of 129.17 mM (Table 1), which was similar to that of rainbow trout (127 mM: Morisawa et al. 1983b) and higher than that of common carp *Cyphmts carpio* (75 mM; Morisawa et al. 1983a). Potassium concentration (27.88 mM) was lower than that of rainbow trout (37.3 mM) and common carp (70.2 mM). Sperm motility was regulated by a similar mechanism in both muskellunge and common carp. Further information is necessary to understand the relationship between ionic composition and regulation of sperm motility. Calcium, magnesium, and chloride concentrations were comparable to those of rainbow trout. Calcium concentration in common carp seminal plasma varied between 2.11-2.88 mM during the sperm production season (Kruger et al. 1984). Osmolality of muskellunge seminal plasma was slightly lower than that of rainbow trout (297 mos-mol/kg) and common carp (302 mosmol/kg). In common carp, Kruger et al. (1984) found seasonal variation in osmolality of seminal plasma. The differences in osmolality between seminal plasma of common carp and muskellunge were not evident at the time of spawning. Osmolality of muskellunge seminal plasma was measured with frozen samples because the osmometer was not available at the time of sample collection. In order to examine the effect of storage on osmolality, the osmolality of seminal plasma from 13 yellow perch was measured both before and after freezing. There was no significant difference in osmolality between fresh and frozen samples ($P > 0.05$, paired *t*-test, unpublished results).

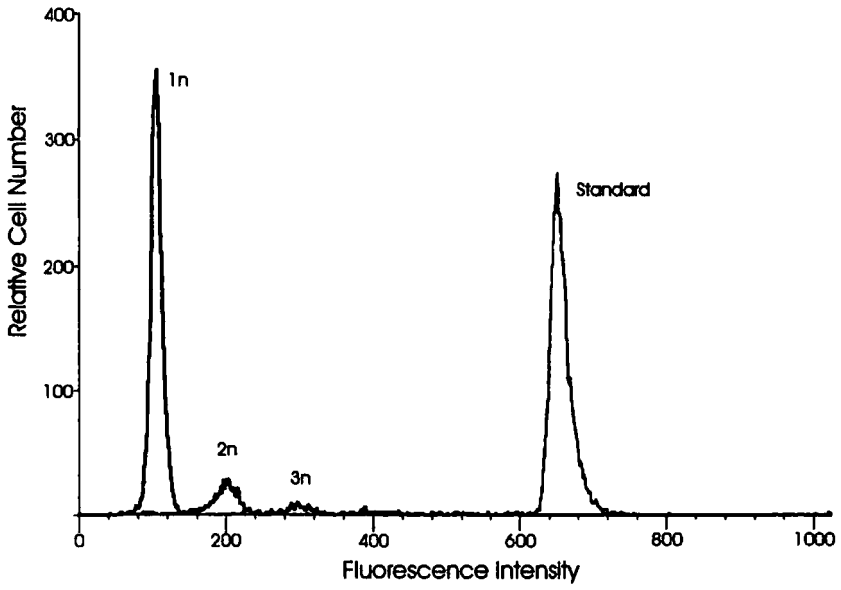

FIGURE 3. — Representative fluorescence distributions of muskellunge sperm and rainbow trout red blood cells. Samples were stained with propidium iodide. Haploid (1n) sperm accounted for 85.3% of the total cells and had a mean fluorescence intensity of 102.7; diploid (2n) sperm (7.9% of the total) had a mean fluorescence intensity of 200.0; triploid (3n) sperm (1.9%) and had a mean fluorescence intensity of 299.5. Rainbow trout red blood cells serving as a standard had a mean fluorescence intensity of 641.8.

Aspartate aminotransferase and Antiproteinase Activity

Aspartate aminotransferase activity of muskellunge sperm extracts was 1,192 ± 196 mU/mg protein without PLP and 2,344 ± 163 mU/mg protein with PLP ($N = 10$). Supplements of exogenous coenzyme in the preincubation mixture produced a significant ($P < 0.001$) increase in AspAT activity (96%). This increase suggested that only partial enzyme activity was measured without PLP. Thus, measuring AspAT activity with PLP was recommended. The AspAT activity of muskellunge sperm extracts appeared to be higher than that of rainbow trout and lake whitefish (Ciereszko and Dabrowski 1994). However, different methods of sperm preparation for the estimation of AspAT activity limit the direct comparison.

Seminal plasma antiproteinase activity was 88.7 ± 38.3 U/L, which was considerably lower than that of rainbow trout (396.0 U/L) and yellow perch (905.2 U/L; Dabrowski and Ciereszko 1994). Dabrowski and Ciereszko (1994) recently demonstrated for the first time the existence of the proteinase inhibitor(s) in seminal plasma of rainbow trout, lake whitefish, and yellow perch. We confirmed the existence of an anti-cod trypsin activity in the seminal plasma of muskellunge. Further studies are required to determine the biological function of this protein.

Sperm DNA Content

Mean fluorescence of muskellunge sperm ($N = 9$) was 0.188 ± 0.008 times that of diploid rainbow trout red blood cells that were used as a standard (Figure 3). Assuming that the internal standard of rainbow trout red blood cells (TRBC) contained 4.66 pg DNA/cell (Johnson et al. 1987; Lockwood and Derr 1992), haploid sperm cells of muskellunge contained 0.88 ± 0.01 pg DNA/cell. However, the DNA content of muskellunge sperm was significantly different ($P <$

0.0001) from half of the DNA content of diploid embryonic cells, which was 0.474 that of TRBC (2.21 pg DNA/cell, unpublished data). The underestimation of DNA content of sperm might be partially explained by the condensed structure of sperm chromatin. Evenson et al. (1986) demonstrated that the DNA stain-ability of spermatozoa was significantly lower than that of diploid cells in propidium iodide and seven other dyes in mouse. Variations of dye binding capacity in different cell populations could cause ambiguity in estimation of the correct DNA content of the species (Shankey 1993; Tiersch and Wachtel 1993). Thus, one should be cautious when choosing sperm as a standard for ploidy identification and estimation of haploid DNA content. We found that two smaller peaks corresponding to 2 and 3 times the haploid sperm occurred in the flow cytometric histograms. Software gating to exclude the sperm doublets did not eliminate these peaks. This suggests the possible existence of 2n and 3n sperm in muskellunge, which may be of great interest in ploidy manipulation of this species. Allen et al. (1986) also reported the existence of peaks (in decreasing abundance) at every ploidy level up to 6n from sperm samples of diploid grass carp *Ctenopharyngodon idella*. They attributed peaks at 3n and up to cell aggregates. The 2n and 3n sperm, if viable, could be sorted and used to produce triploids and tetraploids without the need for chromosomal manipulation, such as heat shock or pressure treatments, that might cause damage to chromosomes and threaten the survival of embryos.

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